(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 January 2003 (30.01.2003)

PCT

(10) International Publication Number
WO 03/008973 A2

(51) International Patent Classification[7]: G01N 33/536, 33/566, 33/577, A01K 67/00, A23K 1/00

(21) International Application Number: PCT/GB02/03315

(22) International Filing Date: 19 July 2002 (19.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0117790.6 20 July 2001 (20.07.2001) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/008973 A2

(54) Title: DIAGNOSTIC MARKER

(57) Abstract: The present invention relates to a non-human animal life stage classification system, particularly, but not exclusively using a physiological marker of an individual non-human animal. The life stage classification can be used to more accurately define the life-stage of companion animals.

DIAGNOSTIC MARKER

The present invention relates to a non-human animal life stage classification system, particularly, but not exclusively using a physiological marker of an individual non-human animal.

The life stage classification can be used to more accurately define the life-stage of companion animals.

Currently, animals are "aged" using approximate equivalent ages to humans. Studies in humans divide individuals into infant, adult and senior life-stage groups without any clear definition of how these divisions are made. Likewise, canine studies have defined Labrador dogs with a mean age of 2.4 years as being young, those with a mean age of 5.8 as middle aged and those with a mean age of 7.1 as old. Alternatively definitions have been presented for German Shepherd dogs as young, adult and old with no clear guidelines of how these groups were defined.

This "ageing" is arbitrary and does not reflect physiological changes which represent changes in the life-stage of the animal. There is a consensus, that the function of the mammalian immune system changes quite dramatically with age. Recent data would suggest that immunosenescence is a more complex process than previously envisaged. Thymic involution, commonly thought to be the major causative factor in age related immune deficiency, may not play as central a role as initially perceived. Some studies have shown that certain aspects of immune function, in particular elements of the more evolutionary distant innate response, seem to be preserved or possibly upregulated well into old age. This suggests that immunosenescence may be more accurately viewed as an immune remodeling process rather than a simple deterioration.

Accordingly, there is a need to define a life-stage classification, for non-human animals. Such a classification system can be used to determine the effects, of various factors, on ageing.

In any life-stage classification, a marker of change is required. The present inventors have determined that a particular marker is a good measure to define life-stages. This marker provides a better life-stage classification than previous markers such as weight or other physical measurements.

Accordingly, the present invention provides, according to a first aspect, the use of a physiological marker to determine the life-stage of a non-human animal.

The present inventors have determined that significant age-related changes in several physiological parameters, reflect an altered functional status of the canine and feline animal. Using these changes, life-stage classifications were identified. This is the first report which has based life-stage classifications on a physiological marker and opens up the potential for designing specific life-stage diets based on changing physiological status with age. The present invention provides a platform to monitor development, maintenance and modulation of the canine or feline physiological system with age.

The present invention provides novel strategies (in particular utilising nutritional intervention) to enhance the ability of the physiological system to cope with stressors (a disorder which has a component of oxidative stress).

These disorders include cancer, ageing, heart disease, atherosclerosis, arthritis, cataracts, inflammatory bowel disease, renal disease, renal failure, neurodegenerative disease or comprised immunity, for example, an animal suffering from an infection.

Using one or more indicators of changing physiological function to define life-stage groups (puppy/kitten, adult and senior) provides the ability to balance nutritional

requirements for a particular life-stage to help maintain optimal physiological function, or help boost a sub-optimal physiological system and potentially reduce the incidence of stressors which are associated with ageing.

The present inventors have identified that changes in the immune status occur at distinct times in the life of caninė and feline animals.

In the present invention, the physiological marker may be one or more indicators of immune status. Such indicators include one or more cell-mediated immune marker, one or more antibody mediated immune marker or one or more innate immune marker.

Particular cell mediated immune markers, according to the present invention, include T-cell markers such as populations of CD3 cells, CD4 cells, CD8 cells, CD21 cells, CD11b cells, granulocytes and lymphocytes. The ratio of CD4:CD8 cells is also a useful marker.

In the first aspect of the invention, the life-stage may be defined by any means. It can be by time (i.e. age) or by division into puppy/kitten, adult and senior areas (or others).

The present invention applies primarily to non-human animals, in particular to companion animals. Typical companion animals include cats and dogs, such as the domestic dog (*Canis domesticus*) and the domestic cat (*Felis domesticus*). All breeds are encompassed in the present invention. Any life-stage classification may cover all species or may be species (i.e. breed) specific. For example, the life stage classification may be for Labrador or German Shepherd dogs, specifically. It may also relate for example to large breed dogs versus small breed dogs.

The second aspect of the present invention relates to a method for determining the life-stage of a non-human animal, the method comprising determining the population

of one of the following T-cells in an individual animal, and with reference to the indicated figure, determining the life stage of the animal:

For a canine animal;

CD3: Figure 1a or Figure 2a
CD4: Figure 1b or Figure 2b
CD8: Figure 1c or Figure 2c
CD4:CD8 ratio: Figure 1d or Figure 2d
CD21: Figure 1e or Figure 2e.

For a feline animal;

CD4: Figure 3
CD8: Figure 4 or Figure 9
CD4:CD8 ratio: Figure 5
Granulocytes: Figure 6
Lymphocytes: Figure 7
CD11b cells: Figure 8.

Significant age-related changes were seen to occur in the T and B parameters measured. CD3 and CD8 were the most significantly affected parameters. Highly significant changes were determined between animals less than 8-12 months of age, between 1-8 years of age and greater than 8 years of age. Accordingly, the following life-stage classification is presented according to the present invention:

Life-stage	Life-stage in years
Puppy/kitten	<8-12 months of age
Adult dog/cat	1-8 years of age
Senior dog/cat	>8 years of age

These life stages have been established on the basis of the following immune markers:

Life-stage	Life-stage in years
Puppy/kitten	CD4:CD8 ratio
Adult dog/cat	CD4:CD8 ratio, or CD3, CD8, or CD21
Senior dog/cat	CD4:CD8 ratio, CD3 or CD8 or CD 21

All preferred features of the first aspect also apply to the second.

A third aspect of the present invention provides a non-human animal life-stage classification system comprising a figure, chart or a table which relates the level of a physiological marker of an individual animal to a particular life stage for the animal.

Examples of figures can be found in any of the Figures 1a, 1b, 1c, 1d 1e, 2a, 2b, 2c, 2d, 2e, 3, 4, 5, 6, 7, 8 and 9.

As for all aspects of the invention, the physiological marker may be any which has been shown to be associated with the ageing process. As well as markers which reflect immune status, others which are suitable include markers of the cell-cycle, DNA repair, antioxidant markers etc.

All preferred features of aspects one and two, also apply to the third.

A fourth aspect of the present invention provides the use of a life-stage classification, according to the third aspect of the invention, to determine the effect of one or more factors on the physiological marker. The physiological marker may be any, particularly those described for the first to third aspects of the invention.

The factor to be determined is not limiting. Suitable factors include: diet, medicine, environmental change (seasons or geographical change), stress etc.

A particular feature of the fourth aspect of the invention is to determine the effect of diet. The term "diet" here includes nutrition and covers all foodstuffs which the animal ingests. It includes the traditional basic foodstuffs, as well as supplements, drinks, snacks, treats etc. The foodstuff includes wet diets, dry diets and semi-moist diets.

In accordance with the present invention, a life-stage classification can be used to determine the effect of diet. In this manner, diet can be identified which has a beneficial effect on the animal. Accordingly diets can be identified which restore declining immune status.

Dietary intervention can thus be provided for a beneficial effect. The diet may need to be life-stage specific. Or, it may be species specific (breed species) such as Labrador dogs or large breed versus small breed specific. The diet may be both life-stage and species (or other) specific. Inter-breed differences in life-span may have a major impact on immune status with age, leading to/supporting different diets for different breeds. Nutritional supplementation (e.g. vitamin C) or combinations of supplements or other diet can be used to maintain optimal immune performance, or rejuvenate age-related reduced/suppressed immune function in cat and dog populations. From a clinical perspective, baseline data from a healthy population can be used for comparative purposes with populations that are clinically immunosuppressed to assess the effectiveness of clinical diets.

All preferred features of the first to third aspects also apply to the fourth.

The fifth aspect of the invention relates to a companion animal life-stage classification as set out in any one of Figures 1a, 1b, 1c, 1d 1e, 2a, 2b, 2c, 2d, 2e, 3, 4, 5, 6, 7, 8 or 9.

A sixth aspect of the invention provides a method of feeding a non-human animal comprising:

(a) measuring a physiological marker of an animal;

(b) determining a life-stage classification for the animal in dependence upon said measurement; and

(c) supplying the animal with a diet which varies according to the life-stage classification.

All preferred features of the first to fourth aspects also apply to the fifth.

A seventh aspect of the invention provides a method of feeding a non-human animal, substantially as hereinbefore described, with reference to one or more of Figures 1a, 1b, 1c, 1d, 1e, 2a, 2b, 2c, 2d, 2e, 3, 4, 5, 6, 7, 8 or 9.

An eighth aspect of the invention provides an animal foodstuff optimised for a specific life-stage classification of a non-human animal, the said classification being determined according to average measurements of a physiological marker made on a sample population of animals of a species or breed by which the feed is to be consumed.

In the eighth aspect of the invention the average measurements of a physiological marker referred to include typical or representative measurements of said physiological marker.

The animal foodstuff according to the eighth aspect of the invention may be optimised for nutrition of an animal of said classification.

The animal foodstuff of the aspect of the invention may be optimised to enhance the physiology of an animal of said classification. Enhancing the physiology may well enable the animal to cope with stresses typical of said classification.

Enhancing the physiology of an animal may include enhancing representative physiological markers of said animal.

An eighth aspect of the invention may comprise an animal foodstuff optimised to enhance the physiology of an animal of said classification to cope with the disorders typical of said classification.

The animal foodstuff according to the eighth aspect of the invention may comprise a dog foodstuff or a cat foodstuff.

The animal foodstuff according to the eighth aspect of the invention may be optimised, or further optimised for animals of a particular size-range, a particular weight or a particular breed.

An animal foodstuff of the eighth aspect of the invention may comprise the foodstuff being packed within a container which carries a visible indicator of said classification for which it is optimised.

According to the present invention, the non-human foodstuffs encompass any project that a non-human animal consumes in its diet. Specifically companion animal or pet animal foodstuffs are included. Thus, the invention covers the standard food products as well as food snacks (for example snack bars, cereal bars, snacks, biscuits and sweet products). Such food snacks may be pet food snacks. The foodstuff may be a cooked product. It may incorporate meat or animal-derived material (such as beef, chicken, turkey, lamb, fish, blood plasma, marrowbone, etc or one or more thereof). The

foodstuff may alternatively be meat-free (preferably including a meat substitute such as soya, maize gluten or a soya product) in order to provide a protein source. The foodstuff may contain additional protein sources such as soya protein concentrate, milk, protein, gluten etc. The foodstuff may also contain a starch source such as one or more grains (e.g. wheat, corn, rice, oats, barley, etc) or may be starch-free. The foodstuffs may incorporate or may be a gelatinised starch matrix. The foodstuff may incorporate one or more types of fibre such as sugarbeet pulp, chicory, coconut endosperm fibre, wheat fibre etc.

The foodstuff may be a dry, semi-moist or a moist (wet) product. Wet food includes food that is usually sold in tins and has a moisture content of 70% to 90%. Dry food includes food having a similar composition but with 5% to 15% moisture, often presented as small biscuit-like kibbles. Semi-moist food includes food having a moisture content of from above 15% up to 70%. The amount of moisture in any product may influence the type of packaging that can be used or is required.

The foodstuff is preferably packaged. In this way, the consumer is able to identify, from the packaging, the ingredients in the foodstuff and confirm that it is suitable for the particular non-human animal in question. The packaging may be metal (usually in the form of a tin or flexifoil), plastic (usually in the form of a pouch or bottle), paper or card. The amount of moisture in any product may influence the type of packaging, which can be used or is required.

The invention is described with reference to the Figures, in which:

Figure 1a shows a scatter plot of relative percentages of CD3 cells in relation to age (years). A linear regression analysis is provided.

Figure 1b shows an equivalent plot, where the cells are CD4 cells.

Figure 1c shows an equivalent plot, where the cells are CD8 cells.

Figure 1d shows an equivalent plot, where the cells are a ratio of CD4:CD8.

Figure 1e shows an equivalent plot, where the cells are CD21 cells.

Figure 2a is a discriminant analysis plot of CD3 cell populations from dogs, divided into those of up to 8 years and those over 8 years.

Figure 2b shows an equivalent plot, where the cells are CD4 cells.

Figure 2c shows an equivalent plot, where the cells are CD8 cells.

Figure 2d shows an equivalent plot, where the cells are CD4:CD8 ratio.

Figure 2e shows an equivalent plot, where the cells are CD21 cells.

Figure 3 shows a scatter plot of relative percentages of CD4 cells in relation to age (years). A linear regression analysis is provided.

Figure 4 shows an equivalent plot, where the cells are CD8 cells.

Figure 5 shows an equivalent plot where the cells are a ratio of CD4:CD8 ratio.

Figure 6 shows an equivalent plot where the cells are granulocytes.

Figure 7 shows an equivalent plot where the cells are lymphocytes.

Figure 8 shows an equivalent plot where the cells are CD11b cells.

Figure 9 is a discriminant analysis of the CD4:CD8 ratio data divided into those up to 8 months and those of over 8 months of age.

The present invention will now be described with reference to the following examples.

Example 1

Materials and methods

Animals

Whole blood specimens were taken from 122 Labrador dogs (71 females and 51 males) ranging from 0.6 years to 14.2 years in order to establish immunological baseline data on age-related changes in peripheral blood leukocyte subsets.

Sample collection

All blood samples were collected into a 1ml potassium EDTA tube for fluorescence activated cell sorting (FACS) analysis.

Leukocyte profile determination

The immune response is composed of a variety of cell-types (leukocytes) with differing functions. Identification of these different leukocyte populations provides a foundation for understanding the basis of the immune response. With the development of monoclonal antibodies (Mab) with reactivity against leukocyte populations, markers for total T-cells (CD3), T-cell subsets (CD4 and CD8), B-cells (CD21) and monocytes (CD14) represent some of the measurable populations.

FACS analysis has been widely used for characterising and quantifying viable sub-populations of white blood cells. FACS analysis involves three steps; Firstly, cells are prepared and incubated with the relevant Mab against a particular surface marker and labelled with a fluorescent reagent (such as fluorescein isothiocyanate (FITC)). Secondly, stained cells are identified and separated by the FACS and the data collected. Thirdly, the collected data are analysed to obtain quantitative information on the relevant cell populations.

Canine leukocyte populations

Relative expression levels of the following immunological cell surface markers and leukocyte populations were determined by using lysed whole blood staining and triple-colour FACS analysis:

CD3	-	Total T-cell marker
CD4	-	T-helper cell marker
CD8	-	T-cytotoxic marker
CD4:CD8 ratio	-	Relative levels of T-helper to T-cytotoxic cells
CD21	-	B-cell marker
CD14	-	Monocyte marker
Lymphocytes	-	Subpopulation of leukocytes
Granulocytes	-	Subpopulation of leukocytes
Monocytes	-	Subpopulation of leukocytes
Lymphocyte viability	-	Measure of live to dead cells
C11b	-	Cell surface marker

Statistical analysis

Values were expressed as percentages of cellular populations for each individual animal. The results were statistically evaluated by linear regression to assess trends over the whole age ranges. Discriminant analysis was used to identify the subgroup of cell surface markers that were most successful at discriminating between the derived life-stage groupings. For differences between life-stages an independent sample t-test was performed on each of the individual cellular populations to identify significant differences between life-stages.

Results

Canine leukocyte populations

The following scatter plots show the relative percentages of different leukocyte populations in relation to age (years). Linear regression analysis was used to identify significant trends in the data obtained. Analysis identified a significant increase in CD3 (R^2=0.06,p<0.008; Fig 1a), significant decrease in CD4 (R^2=0.03, p<0.05; Fig 1b), a significant increase in CD8 (R^2=0.24, p<0.001; Fig 1c), with a corresponding decrease in the CD4:CD8 ratio (R^2=0.15, p<0.001; Fig 1d) with increasing age.

Age groups for discriminant analysis is at 8 years of age

Canine life-stage groups

These results illustrate a significant correlation between ageing and change in relative percentages of several leukocyte groups in the Labrador dog population. Using discriminant analysis to identify leukocyte populations that were most successful at discriminating between the derived life-stage groupings, CD3 (Fig 2a) and CD8 (Fig 2c) defined two statistically distinct groups, adult dogs (0.6 to 8 years) and senior dogs (8+ years), with an overall correct classification of 83% (cross-validated) for both markers.

Discussion

Canine leukocyte populations

Our investigations have demonstrated significant age-related increases in the relative percentages of CD3 and CD8 T-cells, and decreases in CD4 T-cells and the CD4:CD8 ratio in peripheral blood from the dog population.

Significant changes observed in the present study were with T-cell markers (CD3, CD4, CD8, and CD4:CD8 ratio), all of which are associated with the cellular component of the acquired immune system (response of antigen-specific T-cells to antigen, including development of immunological memory). Although some of the R^2

values are particularly low in some cases (Fig 1a, 1b), this is not unexpected as these data represent the natural variability seen in a population when taking independent samples of healthy dogs over an age range of 0.6 to 14.2 years of age.

The CD3 T-cell marker, which defines total T-cells (encompassing both the CD4 and CD8 T-cell subgroups) demonstrated a significant increase in relative percentage with age (Fig 1a). This may partly be a result from a relative increase in CD8 T-cells that co-express the CD3 marker being greater than a relative decrease in CD4 T-cells that express the same marker. Studies in humans have also demonstrated an increase in levels of CD8 T-cells with age suggesting that these cell types correspond to immature T-cells that are unable to attain their full mature functional status due to the age-associated processes highlighted above. Also, when combined with interpretation of data from other canine and human studies which have examined absolute and relative values of total T-cell subsets, it was shown that an increase in percentage of total T-cells is apparent because the absolute numbers of the T-cell subsets are declining at a lower rate.

While the CD3 marker is commonly shared by the CD4 and CD8 T-cells, their functions are very distinct from each other. The function of CD8 T-cells (cytotoxic T-cells) is to identify and destroy host cells that contain intracellular pathogens (e.g. viruses). CD4 T-cells (helper T-cells) are specialised to activate other cells to destroy extracellular pathogens (e.g. bacteria and parasites) and fall into two functional classes: T-helper 1 (TH1) cells which activate monocyte/macrophages to kill bacteria they harbour, and TH2 cells, which activate B-cells to produce antibody.

The significant increase in relative percentage of canine CD8 T-cells (Fig 1c) observed could form part of what is termed the "memory" T-cell population. As mentioned above, production of memory T-cells indicates that the body has reacted to each antigenic stimulus and mounted a response to suppress or eliminate that particular infection. Over time these same physiological responses lead to a progressive

accumulation of (expanded) clones of memory T-cells that allow the individual to respond more quickly to an infection if that particular antigen is encountered again. This supports the finding that levels of CD8 T-cells increase with age.

In conclusion, studies on measuring functional cellular ageing combined with analyses of cells within the ageing individual provides new insights on how modulation of the immune system develops with age. Evidence from the dog population has demonstrated age-related changes in relative percentages of leukocyte populations in peripheral blood. These studies suggest that continuous restructuring occur in distinct leukocyte populations, potentially altering the functional status of the ageing canine immune system. Information from such studies provides the basic platform from which to monitor the plasticity of the immune system, overall maintenance of basal immunity over time, regulation of compensatory functions and modulation of long-term memory. This information will undoubtedly help develop novel strategies (in particular utilising nutritional intervention) to enhance the ability of the immune system to cope with such stressors (as herein before described) during the life of an animal, in particular during the latter stages of an animal's life.

From the information present, it is clear that significant changes do occur in the canine immune status, showing that there is modulation of immune status with age. Using discriminant analysis on the scatter plot information for each of the leukocyte groups, both the CD3 and CD8 T-cell markers defined two statistically distinct life-stage groups, adult dogs below 8 years of age and senior dogs above 8 years of age (Figs 2a, c). Although the CD4:CD8 ratio (Fig 2d) and CD21 (Fig 2e) showed significant differences, the classification was not as high as for CD3 and CD8.
The possible reason for the lower classification level of the CD4:CD8 ratio is because of the negligible difference in the percentage of CD4 cells between the two life-stage groups (Fig 2b). This may be attributed to the reduced number of dogs at the higher end of the age scale (>8 years), but also the percentage of CD4 cells in the senior

group may have reached a basal level just below the level of the adult group, thus eliminating seeing any significant reductions over 8 years of age.

Although the decline in percentage of CD21 cells was not significant (Fig 1e), the significant difference between the adult and senior life-stage groups (Fig 2e), albeit at a lower classification level, may indicate that a greater reduction in percentage of CD21 cells occurs around the 8 years of age level, which in turn would be masked when put in the overall context of the age scatter plot profile.

Such indicators of changing immune status between different life-stages can be used to determine whether an animal is undergoing immune dysfunction, with the consequences being reduced immune surveillance for infections and potential cancer-inducing factors with increasing age. Information of this kind is useful to define nutritional requirements for a particular life-stage to maintain optimal immune function, or to help boost a sub-optimal immune system and potentially reduce the incidence of degenerative disorders.

<u>Example 2</u>

Materials and Methods

Whole blood samples were taken from 288 Domestic Shorthaired cats, 121 males and 167 females ranging from 0.2 to 15.9 years old. All cats were housed in conditions resembling those of pet cats and were maintained on commercially available complete diets.

Samples were analysed using lysed whole blood staining and two-colour flow cytometric analysis. Commercially available monoclonal antibodies were used to identify cell surface markers for CD5, CD4, CD8, B-cells (CD21-like), CD14 and

CD11b. Relative levels of lymphocytes, monocytes and granulocytes were also calculated.

Values were expressed as percentages of cellular populations for each individual animal. Age-related trends were assessed by linear regression analysis. Discriminant analysis and independent sample t-test were used to identify cellular populations for defining life-stage groupings.

Results

Linear regression analysis identified:

- A significant decrease in CD4 (R^2=0.12, p<0.001; Fig. 3).
- A significant increase in CD8 (R^2=0.15, p<0.001; Fig. 4).
- A significant decrease in CD4:CD8 ratio (R^2=0.23, p<0.001; Fig. 5).
- A significant decrease in relative levels of granulocytes (R^2=0.16, p<0.001; Fig. 6).
- A significant decrease in relative levels of lymphocytes (R^2=0.15, p<0.001; Fig 7).
- A significant increase in CD11b (R^2=0.21, p<0.001; Fig 8).

Discriminant analysis of the CD4:CD8 ratio data (Fig. 8) allowed identification of two statistically distinct groups of cats, kittens (0.2 to 0.8 years) and adults (0.8+ years), with an overall correct classification of 77% (cross-validated).

Discussion

Results from this study have demonstrated:

- A significant age-related decrease in the relative percentage of CD4
- A significant age-related increase in the relative percentage of CD8

- A significant age-related decrease in the CD4:CD8 ratio
- A significant age-related increase in the relative percentage of CD11b

CONCLUSIONS

These studies are the first which comprehensively investigate the effects of ageing on leukocyte subsets in healthy cats and furthermore to define life-stages based on immunological status. The life-stages identified in this study highlight the fact that immunological status does change according to age in the Domestic Shorthaired cat. Therefore, it is imperative that age be considered in any study where the interpretation of leukocyte subset data is utilised.

CLAIMS

1. Use of a physiological marker to determine the life-stage of a non-human animal.

2. Use, as claimed in claim 1, wherein the physiological marker is one or more indicators of immune status.

3. Use, as claimed in claim 2, wherein the immune status is one or more of a cell-mediated immune marker, an antibody-mediated immune marker or an innate immune marker.

4. Use, as claimed in any one of claims 1 to 3, wherein the physiological marker is one or more of the following T-cell populations of an individual animal:

CD3
CD8
CD4
CD4:CD8 ratio
CD21
Granulocytes
Lymphocytes
CD11b

5. Use, as claimed in any one of claims 1 to 4, wherein the life-stage is expressed as age.

6. Use, as claimed in any one of claims 1 to 5, wherein the animal is a companion animal.

7. Use, as claimed in claim 6, wherein the companion animal is a cat or a dog.

8. A method for determining the life-stage of a non-human animal, the method comprising determining the population of one or more of the following cell populations in an individual animal and, with reference to the indicated figure, determining the life-stage:

For a canine animal;	For a feline animal;
CD3 : Figure 1a or 2a	CD4 : Figure 3
CD4 : Figure 1b or 2b	CD8 : Figure 4 or 9
CD8: Figure 1c or 2c	CD4: CD8 ratio: Figure 5
CD4: CD8 ratio: Figure 1d or 2d	Granulocytes: Figure 6
CD21: Figure 1e or 2e.	Lymphocytes: Figure 7
	CD11b: Figure 8.

9. A method, as claimed in claim 8, wherein the life-stage is expressed as age.

10. A non-human animal life-stage classification system comprising a chart or a table which relates the level of a physiological marker of an individual animal to a particular life stage for the individual animal.

11. A life-stage classification, as claimed in claim 10, wherein the non-human animal is a companion animal.

12. A life-stage classification, as claimed in claim 11, wherein the companion animal is a cat or a dog.

13. A life-stage classification, as claimed in any one of claims 10 to 12, wherein the physiological marker is immune status.

14. A life-stage classification, as claimed in any one of claims 10 to 13, wherein the immune status is one or more of a cell-mediated immune marker, an antibody-mediated immune marker or an innate immune marker.

15. A life-stage classification as claimed in any one of claims 10 to 14, wherein the immune status is one or more of the following cell populations:

CD3
CD4
CD8
CD4: CD8 ratio
CD21
Granulocytes
Lymphocytes
CD11b

16. Use of a life-stage classification, as claimed in any one of claims 10 to 15 to determine the effect of one or more factors on the physiological marker.

17. Use, as claimed in claim 16, wherein the factor is diet.

18. A companion animal life-stage classification, as hereinbefore described, with reference to one or more of Figures 1a, 1b, 1c, 1d, 1e, 2a, 2b, 2c, 2d, 2e, 3, 4, 5, 6, 7, 8 or 9.

19. A method of feeding a non-human animal comprising:

(a) measuring a physiological marker of an animal;

(b) determining a life-stage classification for the animal in dependence upon said measurement; and

(c) supplying the animal with a diet which varies according to the life-stage classification.

20. A method as claimed in claim 19, wherein wherein the physiological marker is one or more indicators of immune status.

21. A method as claimed in claim 20, wherein the immune status is one or more of a cell-mediated immune marker, an antibody-mediated immune marker or an innate immune marker.

22. A method as claimed in any one of claims 19 to 21, wherein the physiological marker is one or more of the following cell populations of an individual animal:

CD3
CD8
CD4
CD4:CD8 ratio
CD21
Granulocytes
Lymphocytes
CD11b

23. A method as claimed in any one of claims 19 to 22, wherein the animal is a companion animal.

24. A method as claimed in claim 23, wherein the companion animal is a cat or a dog.

25. A method of feeding a non-human animal, substantially as hereinbefore described, with reference to one or more of Figures 1a, 1b, 1c, 1d, 1e, 2a, 2b, 2c, 2d, 2e, 3, 4, 5, 6, 7, 8 or 9.

26. An animal foodstuff optimised for a specific life-stage classification of a non-human animal, the said classification being determined according to average measurements of a physiological marker made on a sample population of animals of a species or breed by which the feed is to be consumed.

27. An animal foodstuff as claimed in claim 26, optimised for nutrition of an animal of said classification.

28. An animal foodstuff as claimed in claim 26, optimised to enhance the physiology of an animal of said classification.

29. An animal foodstuff as claimed in claim 26, optimised to enhance the physiology of an animal of said classification to cope with disorders typical of the said classification.

30. An animal foodstuff as claimed in any one of claims 26 to 29 comprising a dog foodstuff.

31. An animal foodstuff as claimed in any one of claims 26 to 29 comprising a cat foodstuff.

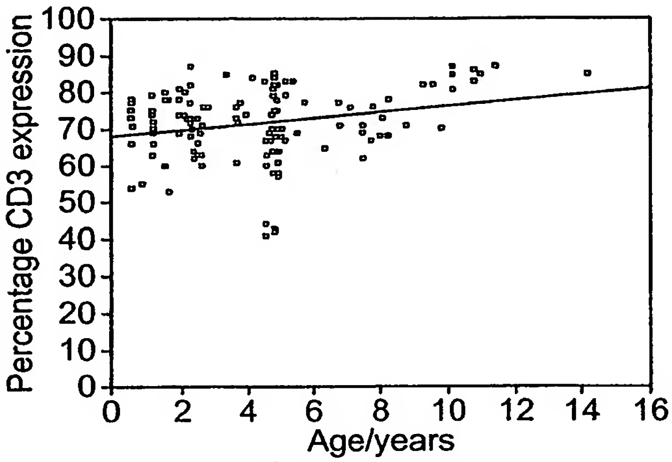
32. An animal foodstuff as claimed in any one of claims 26 to 29 comprising dry, wet or semi-moist foodstuff.

33. An animal foodstuff as claimed in any one of claims 26 to 32 further optimised for animals of a particular size-range.

34. An animal foodstuff as claimed in any one of claims 26 to 32 further optimised for animals of a particular weight.

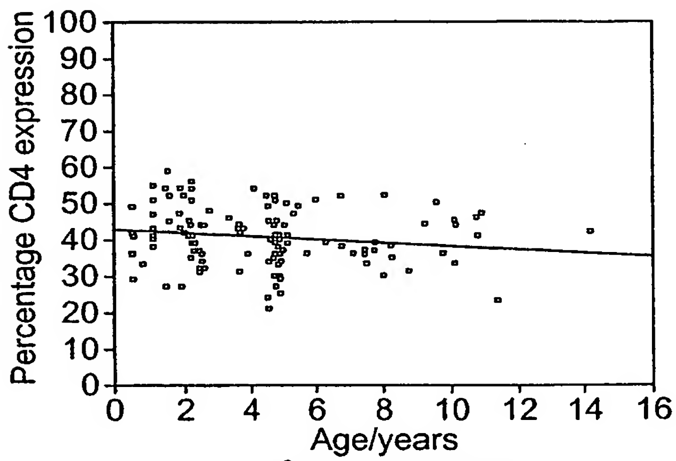
35. An animal foodstuff as claimed in any one of claims 26 to 32 further optimised for animals of a particular breed.

36. An animal foodstuff as claimed in any one of claims 26 to 35, the foodstuff being packed within a container which carries a visible indicator of the said classification for which it is optimised.



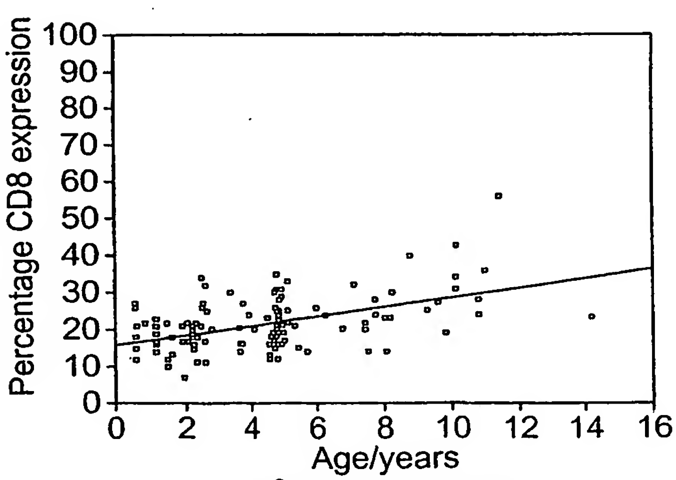
R^2=0.06 (P<0.008)

FIG. 1a - CD3



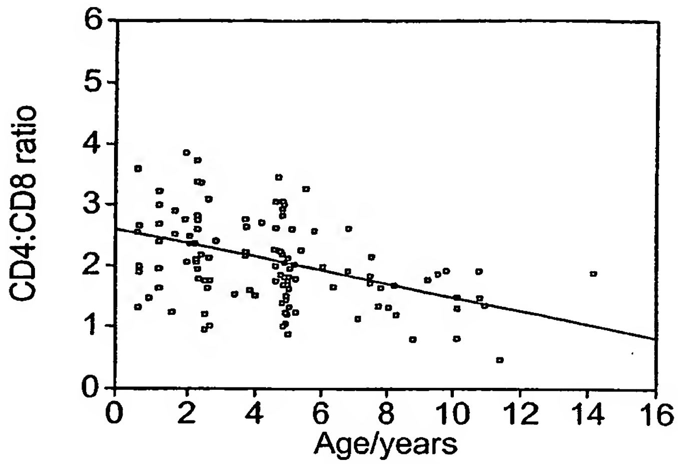
R^2=0.03 (P<0.05)

FIG. 1b - CD4



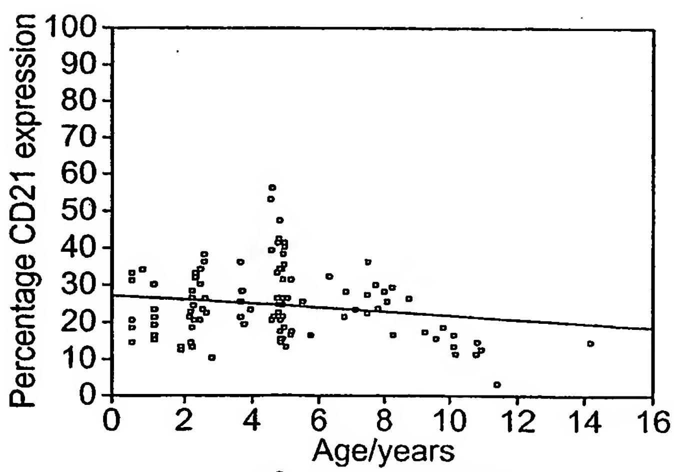
R^2=0.24 (P<0.001)

FIG. 1c - CD8



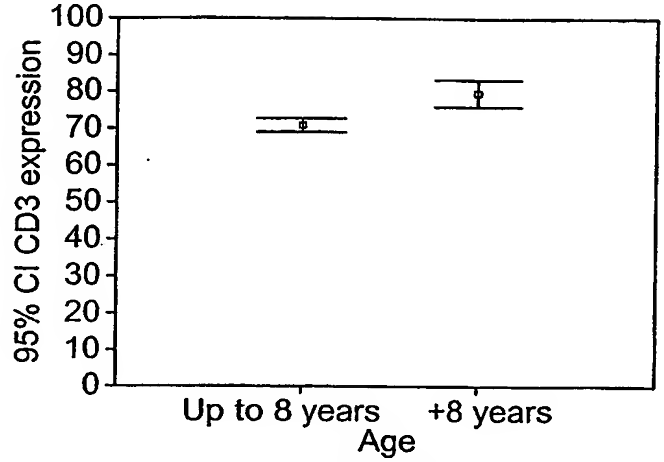
R^2=0.15 (*P*<0.001)

FIG. 1d - CD4:CD8 ratio



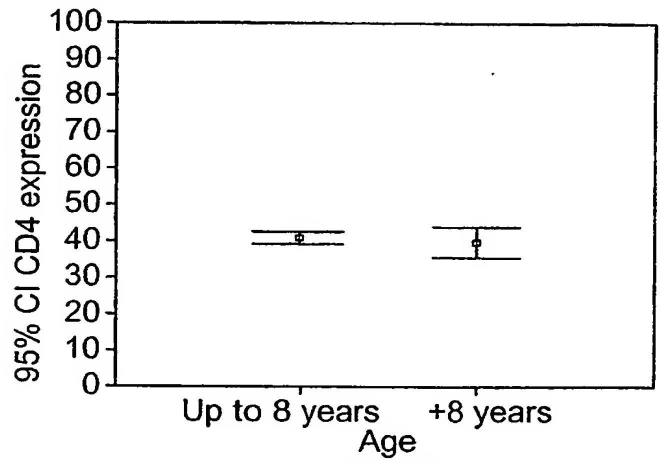
R^2=0.03 (*P*<0.082)

FIG. 1e - CD21



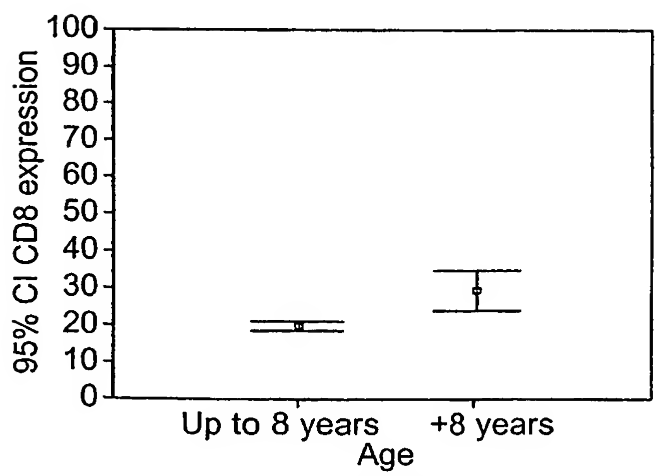
$P<0.001$

FIG. 2a - CD3



$P<0.587$

FIG. 2b - CD4



$P<0.002$

FIG. 2c - CD8

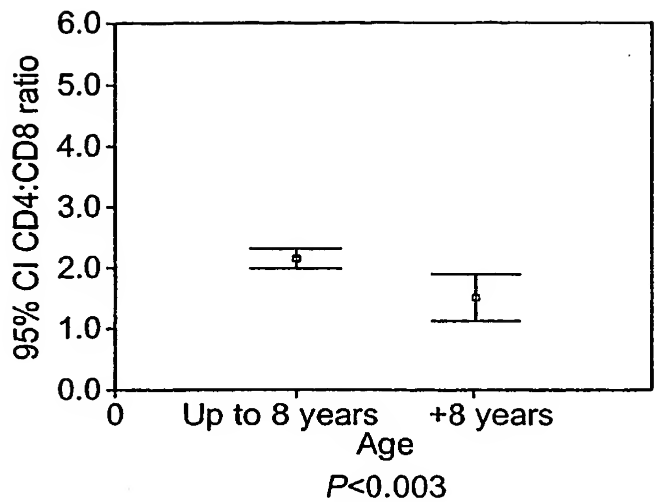


FIG. 2d - CD4:CD8 ratio

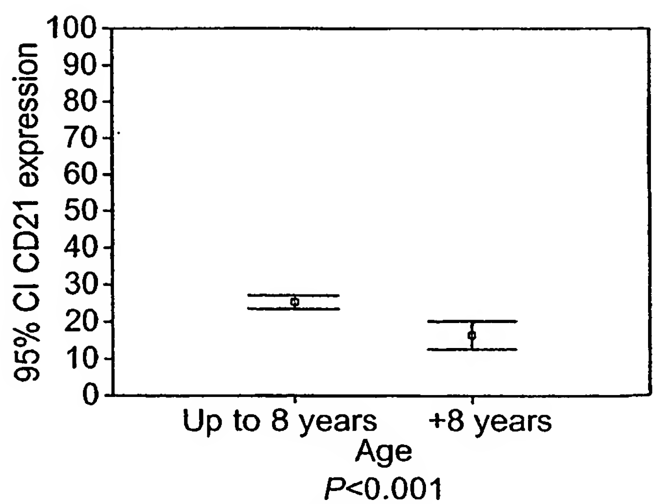
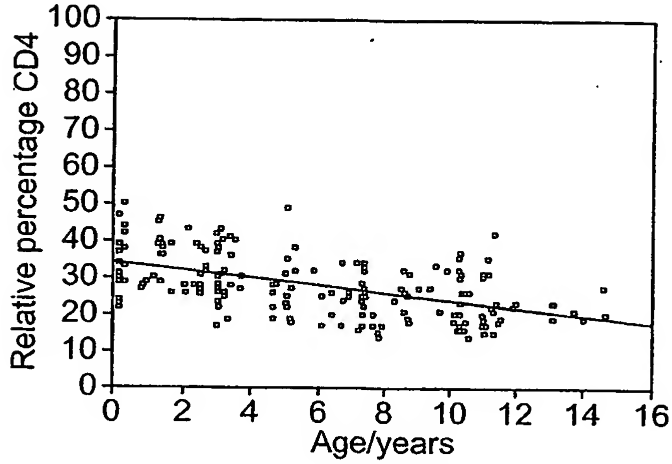
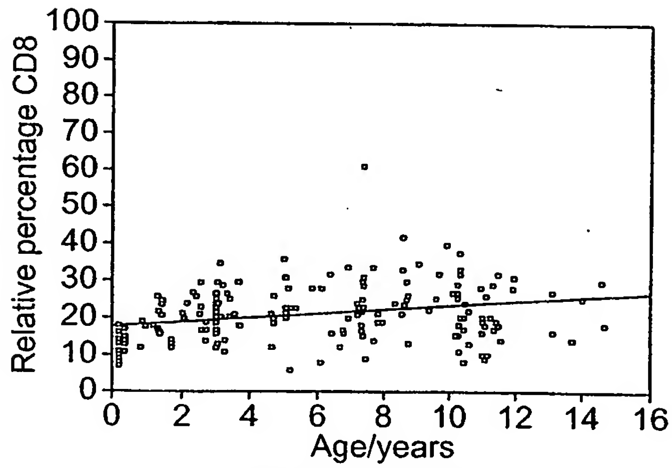


FIG. 2e - CD21



(R2=0.12, p<0.001)

FIG. 3 - CD4



(R2=0.15, p<0.001)

FIG. 4 - CD8

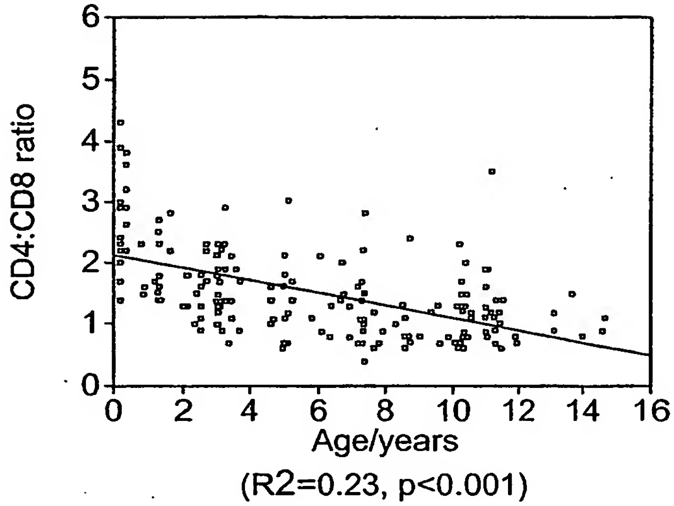


FIG. 5 - CD4:CD8 ratio

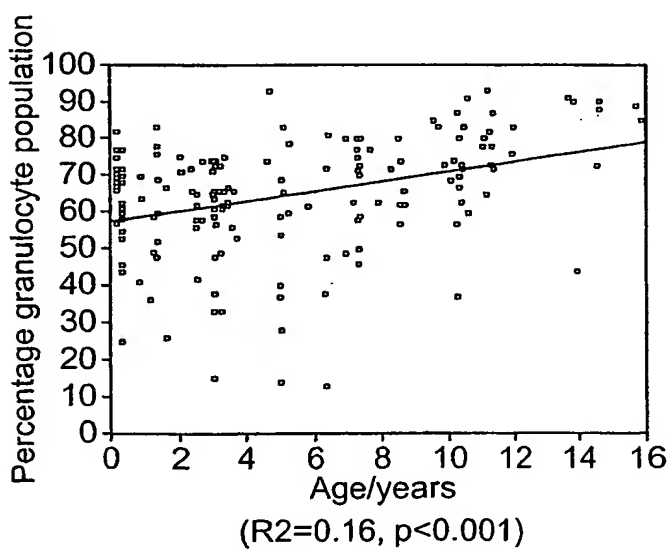


FIG. 6 - Granulocytes

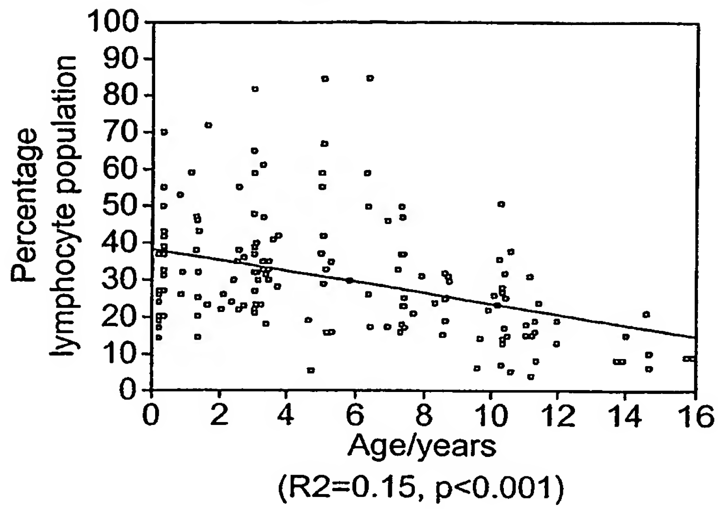


FIG. 7 - Lymphocytes

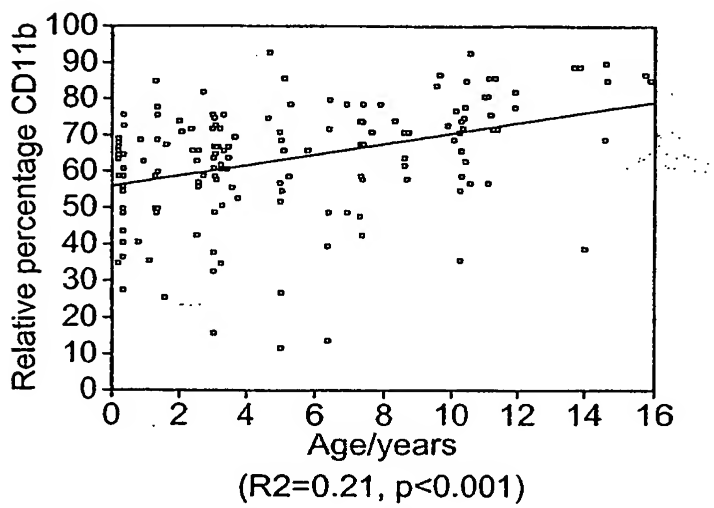


FIG. 8 - CD11b

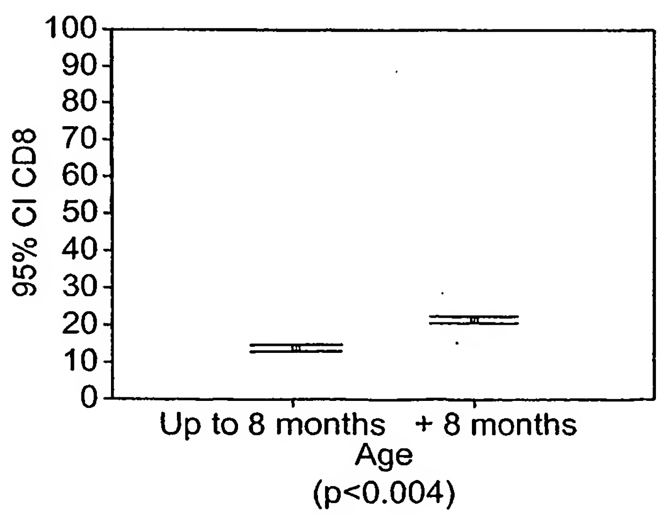


FIG. 9 - CD8

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 January 2003 (30.01.2003)

PCT

(10) International Publication Number
WO 03/008973 A3

(51) International Patent Classification[7]: G01N 33/536, 33/566, 33/577, A01K 67/00, A23K 1/18, G01N 33/569

(21) International Application Number: PCT/GB02/03315

(22) International Filing Date: 19 July 2002 (19.07.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0117790.6 20 July 2001 (20.07.2001) GB

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(74) Agents: **CORNISH, Kristina, Victoria, Joy** et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report: 8 May 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** DIAGNOSTIC MARKERS FOR DETERMINATION OF THE LIFE STAGE OF A NON-HUMAN ANIMAL AND CLASSIFICATION SYSTEM BASED THEREON

(57) **Abstract:** The present invention relates to a non-human animal life stage classification system, particularly, but not exclusively using a physiological marker of an individual non-human animal. The life stage classification can be used to more accurately define the life-stage of companion animals.

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INTERNATIONAL SEARCH REPORT

Intern	l Application No
PCT/GB 02/03315	

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/536 G01N33/566 G01N33/577 A01K67/00 A23K1/18
G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HEATON PAUL R ET AL: "Assessing age-related changes in peripheral blood leukocyte phenotypes in domestic shorthaired cats using flow cytometry." JOURNAL OF NUTRITION, vol. 132, no. 6 Supplement S2, June 2002 (2002-06), pages 1607S-1609S, XP001121945 June, 2002 ISSN: 0022-3166 the whole document page 1	1-9,16, 17,19-25

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2003	28/03/2003

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/03315

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HOSOKAWA HIDEAKI ET AL: "Rapid accumulation of fluorescent material with aging in an oxygen-sensitive mutant mev-1 of Caenorhabditis elegans." MECHANISMS OF AGEING AND DEVELOPMENT, vol. 74, no. 3, 1994, pages 161-170, XP009004347 ISSN: 0047-6374 p. 162, lines 6 - 9 ---	1
X	DATABASE MEDLINE 'Online! November 1995 (1995-11) USPENSKY I: "Physiological age of ixodid ticks: aspects of its determination and application." Database accession no. NLM8551497 XP002228272 abstract the whole document & JOURNAL OF MEDICAL ENTOMOLOGY. UNITED STATES NOV 1995, vol. 32, no. 6, November 1995 (1995-11), pages 751-764, ISSN: 0022-2585 ---	1
X	DAWSON HARRY D ET AL: "Chronic marginal vitamin A status reduces natural killer cell number and function in aging Lewis rats." JOURNAL OF NUTRITION, vol. 129, no. 8, August 1999 (1999-08), pages 1782-1790, XP002228271 ISSN: 0022-3166 the whole document ---	1-9,16, 17,19-25
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; August 1999 (1999-08) BORTNICK SANDRA J ET AL: "Lymphocyte subsets in neonatal and juvenile cats: Comparison of blood and lymphoid tissues." Database accession no. PREV199900510579 XP002228273 abstract & LABORATORY ANIMAL SCIENCE, vol. 49, no. 4, August 1999 (1999-08), pages 395-400, ISSN: 0023-6764 ---	1-9
X	US 6 156 355 A (BENNETT JEFFREY P ET AL) 5 December 2000 (2000-12-05) col. 1, lines 21 - 65 --- -/--	19-36

INTERNATIONAL SEARCH REPORT

Intern l Application No
PCT/GB 02/03315

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 01 58271 A (JUVENON INC) 16 August 2001 (2001-08-16) p. 1, line 9 - p. 2, line 15, p. 3, line 33 - p. 4, line 2, claims 1 - 7	19-36

INTERNATIONAL SEARCH REPORT

In ıtional application No.
PCT/GB 02/03315

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10-15,18
because they relate to subject matter not required to be searched by this Authority, namely:

   Rule 39.1(v) PCT - Presentation of information

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 02/03315

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 6156355	A	05-12-2000	NONE			
WO 0158271	A	16-08-2001	EP	1250052	A1	23-10-2002
			WO	0158271	A1	16-08-2001
			US	2001043983	A1	22-11-2001